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71 Applicant: **INDENA S.p.A.**
Via Ripamonti, 99
I-20141 Milano (IT)

72 Inventor: **Bombardelli, Ezio**
Via Ripamonti, 99
I-20141 Milano (IT)
Inventor: **Morazzoni, Paolo**
Via Ripamonti, 99
I-20141 Milano (IT)

74 Representative: **Minoja, Fabrizio**
Studio Consulenza Brevettuale,
Via Rossini, 8
I-20122 Milano (IT)

54 Formulations containing carotenoids and procarotenoids combined with polyphenols in the prevention of the damages due to an abnormal production of free radicals.

57 The present invention relates to novel formulations and combinations of lipophilic and hydrophilic antioxidants and the use thereof in the therapeutic, foodstuff, dietetic, and cosmetic fields. These formulations are based on the use of carotenoids, procarotenoids and derivatives thereof with polyphenols of catechic and flavanolignane structures. Said formulations, containing a lipophilic antioxidant and an hydrophilic one at fixed ratios, can be used in the prevention of physiopatological conditions related at least partially to an over-production of free radicals, particularly aging, atherosclerosis and cancer.

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The present invention relates to novel formulations and combinations of lipophilic and hydrophilic antioxidants and the use thereof in the therapeutic, foodstuff and cosmetic fields. These formulations are based on the use of carotenoids, procarotenoids (and derivatives thereof) with polyphenols of catechic and flavanolignane structures, both pure and contained in extracts.

5 It is well established in literature that the administration of vitamin E, β -carotene, lycopene and ubiquinol (Coenzyme Q 10) through the diet or in pharmaceutical and nutritional formulations significantly decreases the incidence of cardiovascular diseases, and also it seems to play an important role in the prevention of some tumours. As far as the anti-atherosclerotic activity of carotenoids is concerned, according to recent studies, the capability of carotenoids to prevent lipoproteins oxidation (therefore
10 interfering with the vascular uptake thereof) appears to be one of the most important mechanisms. In fact, carotenoids are incorporated physiologically in low density lipoproteins (LDL) and, preventing the oxidation thereof, they effectively counteract the primum movens of the atherosclerotic damage, which occurs mainly to the detriment of endothelial tissue [1-4].

Carotenoids are effective antioxidants at the cell level, their function taking place particularly on the cell
15 in active proliferation, in which the frequency of genetic error is higher. In support of carotenoids bioavailability, the preventive action thereof, after oral administration, against skin damages due to ultraviolet radiations, which are known to give rise to oxygen radicals, as well as the role thereof at the peripheral level on the eye functions, are well known.

Polyphenol substances having a catechic structure of the dimeric and oligomeric types are widely used
20 in cardiovascular therapy and in ophthalmology due to their action on large and small calibre vessels. These natural polyphenols exert in fact a beneficial modulating action on capillary fragility and permeability, as well as on the protection of endotheliums. Recent literature agrees in considering the antioxidant activity an important mechanism at the base of the biological effect of said compounds.

Flavanolignanes, among which are silymarin and its three main components (silybin, silydianin and
25 silychristin), are characterized by a marked antiradical power at the hepatic and peripheral levels. As recently proved by the Applicant for the procyanidole oligomers extracted from Vitis Vinifera [5] and by other authors for a number of polyphenolic substances extracted from different vegetable sources [6, 7], another interesting biological property of this series of compounds is that they have a remarkable antimutagenic action. Such a characteristic, which is partly connected with the antioxidant effect, takes
30 place both on spontaneous mutagenesis and on the one induced either by ultraviolet radiations or by mutagenic products such as medicaments or atmospheric pollutants.

The action sites of the polyphenolic substances are very different from those of carotenoids and analogues thereof, therefore the concomitant administration of lipophilic and hydrophilic compounds gives rise to an unexpected, surprising advantage from the biological point of view.

35 Carotenoids such as vitamin E, β -carotene, lycopene, coenzyme Q10 and isomers thereof proved to be very effective antiradicalic compounds against the activated forms of oxygen, both in the conventional tests of lipid peroxidation, such as the xanthine-xanthine oxidase system, and in a very selective model, recently prepared by the Applicant, which involves lipid peroxidation of an unsaturated phospholipid in an aqueous medium by means of ultrasounds [8]. In the same tests, the polyphenols of the invention turned out to have
40 the same or a higher activity than the prior art compounds.

The results obtained from these tests, using some polyphenolic substances and the lipophilic antioxidants Vitamin E and lycopene, are summarized in Tables 1 and 2.

Table 1

| Scavenger activity against the OH \cdot hydroxy radical - Inductive phase. | |
|--|------------------------|
| Substances | Cl ₅₀ |
| Proanthocyanidin A2 | 1.7 X 10 ⁻⁷ |
| 90% Procyanidole oligomers from <u>Vitis vinifera</u> | 1.1 X 10 ⁻⁷ |
| Silymarin | 1.2 X 10 ⁻⁷ |
| Vitamin E | 1.5 X 10 ⁻⁶ |
| Lycopene | 2.1 X 10 ⁻⁶ |

Table 2 - Scavenger activity against R^\bullet , ROO^\bullet lipid radicals. Propagation phase.

| 5 | Substances | CI_{50} |
|----|--|----------------------|
| | Proanthocyanidin A2 | 4.0×10^{-7} |
| 10 | 90% Procyanidole oligomers from <u>Vitis vinifera</u> | 5.1×10^{-7} |
| | Silymarin | 5.2×10^{-7} |
| 15 | Vitamin E | 1.0×10^{-7} |
| | Lycopene | 1.8×10^{-7} |
| 20 | Lycopene + Proc. olig. from <u>Vitis vinifera</u> 1:4 | 1.2×10^{-9} |

It has surprisingly been found, and it is one object of the present invention, that the combination of an hydrophilic antioxidant with a lipophilic one exerts an antioxidant action far greater than that of the single compounds tested at equal concentrations (Table 2).

This finding was confirmed in another *in vitro* model which uses human fibroblasts stimulated with zymosan and evaluation of the peroxidative process by chemoluminescence. The combination of lycopene and silymarin, for example, proved to have an antioxidant effect surprisingly more marked than the single components (Fig. 1).

The same results were obtained using other combinations of lipophilic antioxidants (ubidecarenone, vitamin E) and hydrophilic antioxidants (oligomers extracted from Vitis vinifera).

The results obtained in the *in vitro* models using pure lycopene were subsequently confirmed by *in vivo* models in which the use of combinations of lipophilic antioxidants and hydrophilic antioxidants gave an effective, unpredictable improvement on the experimental damages in which free radicals play an important role.

As a control, we selected in fact an inflammatory process model in which an irritating agent (phorbol ester) is used, which triggers off lipid peroxidation at the level of biological membranes and subsequently propagates through a radical chain reaction.

Table 3 shows the data of the antioedemigenic activity in the mouse, obtained with silymarin, silymarin combined with a lipophilic fraction having a high lycopene content (5%), the fraction being prepared by hexane extraction of dried skins of Licopersicum aesculentum and the fraction with lycopene. Data are expressed as percent decrease of the oedema measured at the 6th hour after the induction.

Table 3

| Anti-oedemigenic activity of silymarin, lycopene and combinations thereof on the phorbol ester oedema in the mouse. | | | |
|---|---------------------------------|-----------------|------------|
| Substances | Dose $\mu\text{g}/\text{mouse}$ | Oedema | % decrease |
| Controls | -- | 8.1 ± 0.2 | -- |
| Silymarin | 300 | 6.3 ± 0.2 | 22.2 NS |
| | 150 | 7.3 ± 0.4 | 9.9 NS |
| 5% Lycopene | 200 | 6.8 ± 0.3 | 16.0 NS |
| | 100 | 7.5 ± 0.4 | 12.4 NS |
| 5% Lycopene + Silymarin | 200 + 100 | $1.2 \pm 0.2^*$ | 86.4 |
| | 100 + 50 | $5.1 \pm 0.0^*$ | 37.0 |
| average \pm SD * $p < 0.01$ | | | |

All the above reported data show that the combination of the two antioxidants of different polarity increases by far the effectiveness of the single compounds. Most likely, such synergism is due to the concomitant action on more radical species which gives rise to a nearly complete blockage of the peroxidative process.

The same phenomenon takes place when the antimutagenic effect of these combinations is checked, by which effect the antioxidant component plays a paramount role and which is of paramount importance due to the implications in the prevention of cancer and cardiovascular pathologies.

By way of example, in Table 4 the effect of polyphenols extracted from green tea, of lycopene and of a combination thereof is reported.

Table 4

| Anti-mutagenic activity of procyanidole oligomers extracted from green tea, lycopene and a combination thereof on the frequency of spontaneous mutation in <i>Salmonella typhimurium</i> . | | | | |
|--|----------|----------|-----------|-----------|
| Substance mg/plate | TA98 | | TA100 | |
| | -S9 | + S9 | -S9 | + S9 |
| 0 | 1.0 (41) | 1.0 (48) | 1.0 (163) | 1.0 (133) |
| Proc. olig. | | | | |
| 1.0 | 1.0 | 0.8 | 0.9 | 1.0 |
| 2.5 | 1.0 | 0.7 | 1.1 | 1.0 |
| 5.0 | 0.5 | 0.5 | 0.7 | 0.9 |
| Lycopene | | | | |
| 0.2 | 1.0 | 0.9 | 0.9 | 0.8 |
| 0.4 | 0.8 | 0.8 | 1.0 | 0.9 |
| 0.5 | 0.7 | 0.7 | 0.9 | 0.8 |
| Proc. olig. + Lycopene | | | | |
| 0.50 + 0.10 | 0.6 | 0.5 | 0.7 | 0.7 |
| 1.25 + 0.20 | 0.5 | 0.5 | 0.7 | 0.8 |
| 2.50 + 0.25 | 0.4 | 0.3 | 0.7 | 0.7 |

As the polyphenolic fraction from green tea, a product is used having an 85% polyphenol titre, expressed as epigallocatechin gallate, and a low caffeine content (<0.3%). Using mixtures of the lycopene fraction with tea polyphenols, inhibition of about 50% of spontaneous mutation was obtained in the Ames test (*Salmonella typhimurium* TA-100 and TA-98).

Such an effect turned out to be independent of the metabolic activation (-S9 or + S9). Analogous effects are obtained on *Saccharomyces cerevisiae* strains, where the effect on spontaneous mutation was controlled at the nuclear level and, even more important, at the level of mitochondrial information.

The ratios of the two fractions can vary from 1 part of polyphenol to 1 part of lipophilic antioxidant, depending on the field of application of the different formulations.

As excipients for use in these formulations, particularly important are phospholipids, both the natural and the synthetic ones, and the raw lecithins having different phospholipid contents. Phospholipids are a particularly suitable carrier to promote adsorption of polyphenolic substances both of catechic and flavanolignane nature. The preferred administration forms of these combinations are soft gelatin capsules, but hard gelatin capsules, cellulose or other matrixes as well as suppositories and transdermal forms can also be envisaged. The following examples further illustrate the invention.

Example I - Formulation containing silymarin and lycopene in peanut oil

200 mg of lipophilic extract of *Licopersicum aesculentum* containing 5% of lycopene are mixed with 50 mg of silymarin, 50 mg of natural soy phosphatidylcholine and 50 mg of peanut oil; the products are encapsulated in soft gelatin capsules. The dose can vary from 1 to 5 capsules daily.

Example II - Formulation containing procyanidole oligomers and lycopene in peanut oil

200 mg of lipophilic extract of *Licopersicum aesculentum* y containing 5% of lycopene are mixed with 80 mg of procyanidole oligomers from *Vitis vinifera*, 50 mg of natural soy phosphatidylcholine and 50 mg of peanut oil; the products are encapsulated in soft gelatin capsules. The dose can vary from 1 to 5 capsules daily.

Example III - Formulation containing Ubidecarenone and procyanidole oligomers

20 mg of ubidecarenone are mixed with 20 mg of vitamin E and 80 mg of procyanidole oligomers from green tea; this mixture is dispersed in 100 mg of peanut oil containing 50 mg of purified soy phospholipids.
 5 The suspension can be encapsulated in soft gelatin capsules or adsorbed on suitable excipients for the preparation of tablets. The dose can vary from 1 to 3 capsules daily.

Example IV - Formulation containing ubiquinol (reduced ubidecarenone), unsaturated phospholipids and Proanthocyanidin A2

10 20 mg of ubiquinol are mixed with 100 mg of soy phosphatidylcholine and dissolved in 100 mg of peanut oil. This solution is added with 80 mg of Proanthocyanidin A2. The suspension can be encapsulated in soft gelatin capsules. The dose can vary from 1 to 3 capsules daily.

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45 Claims

1. Pharmaceutical, foodstuff, dietetic or cosmetic compositions containing as the active principle a lipophilic antioxidant in combination with an hydrophilic antioxidant.
- 50 2. Compositions according to claim 1 wherein the lipophilic antioxidant is selected from the group of carotenoids, procarotenoids and derivatives thereof.
3. Compositions according to claim 2 wherein the lipophilic antioxidant is selected from vitamin E, β -carotene, lycopene, ubidecarenone, ubiquinol.
- 55 4. Compositions according to any one of claims 1-3 wherein the hydrophilic antioxidant is selected from the group of polyphenols of catechic or flavanolignane structure.

5. Compositions according to claim 4 wherein the hydrophilic antioxidant is selected from silymarin or one of the components thereof (silybin, silydianin, silychristin and isosilybin), proanthocyanidin A2, pro-cyanidole oligomers extracted from Vitis vinifera, Camellia sinensis, Aesculus hippocastanum, Ginkgo biloba, Cardus marianum.
- 5
6. Compositions according to any one of claims 1-5 containing as excipients natural or synthetic phospholipids, including raw lecithins having different phospholipid contents.
7. Compositions according to any one of the above claims, in form of soft or hard gelatin capsules, tablets, suppositories or transdermal forms.
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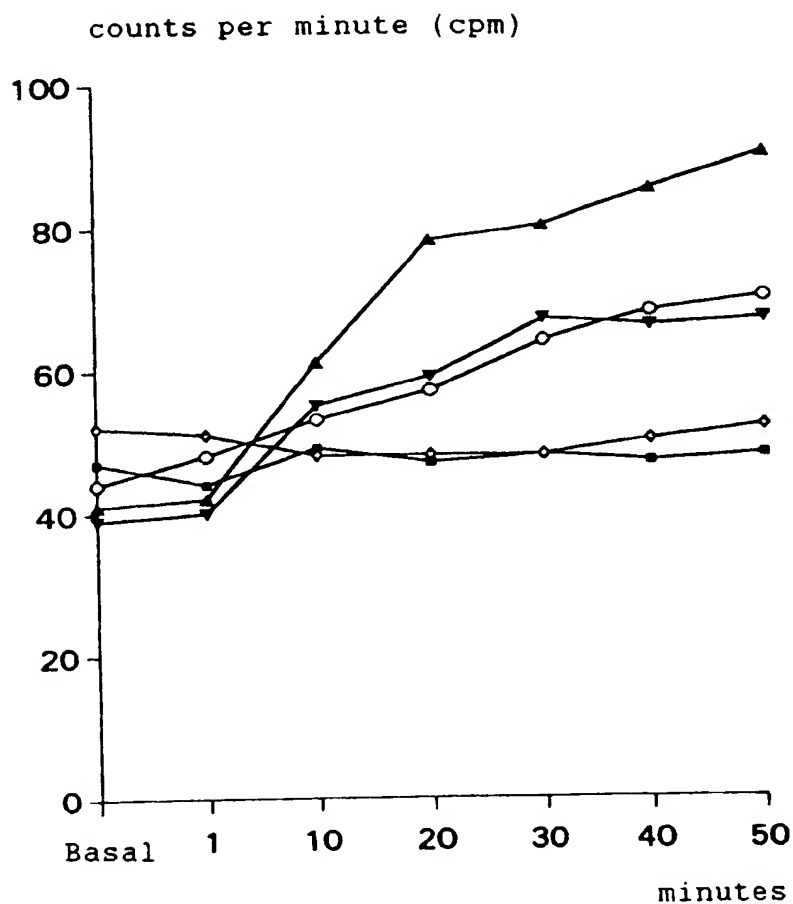
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Figure 1 - Antioxidant activity of silymarin, lycopene and a combination thereof on peroxidation in a culture of fibroblasts stimulated with zymosan



- ▲ : controls stimulated with zymosan
- ◇ : non-stimulated controls
- : silymarin 2.5 mg + lycopene 2.5 mg + zymosan
- : silymarin 5 mg + zymosan
- ▼ : lycopene 5 mg + zymosan

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(71) Applicant: **INDENA S.p.A.**
20141 Milano (IT)

(72) Inventors:
• **Bombardelli, Ezio**
I-20141 Milano (IT)
• **Morazzoni, Paolo**
I-20141 Milano (IT)

(74) Representative: **Minoja, Fabrizio**
Studio Consulenza Brevettuale,
Via Rossini, 8
20122 Milano (IT)

(54) **Formulations containing carotenoids and procarotenoids combined with polyphenols in the prevention of the damages due to an abnormal production of free radicals**

(57) The present invention relates to novel formulations and combinations of lipophilic and hydrophilic antioxidants and the use thereof in the therapeutic, foodstuff, dietetic, and cosmetic fields. These formulations are based on the use of carotenoids, procarotenoids and derivatives thereof with polyphenols of catechic and flavanolignane structures. Said formulations, containing a lipophilic antioxidant and an hydrophilic one at fixed ratios, can be used in the prevention of physiopatological conditions related at least partially to an over-production of free radicals, particularly aging, atherosclerosis and cancer.

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EUROPEAN SEARCH REPORT

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| The present search report has been drawn up for all claims | | | |
| Place of search BERLIN | | Date of completion of the search 5 August 1996 | Examiner Alvarez Alvarez, C |
| <p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone V : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application I : document cited for other reasons</p> <p>& : member of the same patent family, corresponding document</p> | | | |

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EUROPEAN SEARCH REPORT

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| DOCUMENTS CONSIDERED TO BE RELEVANT | | | |
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| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int. CL.6) |
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| The present search report has been drawn up for all claims | | | TECHNICAL FIELDS SEARCHED (Int. CL.6) |
| Place of search BERLIN | | Date of completion of the search 5 August 1996 | Examiner Alvarez Alvarez, C |
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